| TION | AFTER CORRECTION | |
|---|---|---|
| r | SLOPE | r |
| .99 | 0.80 | 0.99 |
| .99 | 0.99 | 0.99 |
| .99 | 0.89 | 0.99 |
| .99 | 0.98 | 0.99 |
| .99 | 0.92 | 0.99 |
| | 0.92 (0.08) | |

unchanged before and after correction (0.94 sed by 70% (0.26 vs 0.08). These results luced the bias when MB-MASS calibration er's recommended diluents. The evolving ays may be one in which each manufacturer e of purified CK-MB provided by a national to be determined.

## AN ALTER RESULTS FOR SERUM L ($E_2$), Gerald Knee, Gregory Fossum, , (Dept. Obstetrics and Gynecology, ia, PA, 19107)

ay techniques have been developed to es in serum without the need to extract t assay results on unextracted samples the serum sample. To determine the serum estradiol ($E_2$) concentrations, women (n=20) with regular, cyclic, l cycles before and after ingestion of a amples were collected during the late nd 3 hours after a high lipid meal. All ying 2 different assay systems: SR1 $E_2$ ono-Baker Diagnostics, Allentown, PA IA (Diagnostic Products Corporation, , we reported that the results obtained ll (DPC to SR1 (n=292) y = -22.80 + $E_2$ concentrations in serum obtained of the lipid meal were compared, the techniques for the pre-meal samples obtained with DPC and SR1 were 128 On the other hand, we noted that the R1 for the post-meal samples was 76 ined with DPC was 106 pg/mL (16% ears to effect the results obtained with ith SR1 were 18% lower than those ingestion of a meal high in lipid content lts obtained for serum concentration of with the SR1 system. Thus, it appears ence the results obtained to different serum lipemia and the assay technique nples should be obtained in a fasting

## N ASSAY FOR ACETAMINOPHEN ON SYSTEM. Chan Oh, Julie Kim, Josephine ng (Beckman Instruments, Inc., Brea, CA

ric immunoassay for serum acetaminophen an Synchron CX®5 system, an automated tions can be used in the quantitation of the n features an antibody, latex-avidin particle tive as the trigger reagent. The other he antibody as the trigger, and a conjugate x-avidin particle and biotin-drug. Latex gents are mixed, and results in increase in the specimen, inhibits the agglutination by duction of turbidity. Thus, the rate of at 340 nm, is inversely proportional to the specimen. Calibrators are human serum

its were similar and reproducible over the precision studies yielded within-run CV's cross the assay range. Correlation (N = 52 polarization (FPIA as reported in the 0.97x + 1.9 (theophylline method); r = Serial dilutions of samples throughout the ase to dilutions were quantitative error of -glucuronide, -sulfate, -conjugate and -mer- e. Assay interference from bilirubin (30 ycerides (1000 mg/dL) were insignificant

## A PARTICLE-BASED BIOTIN-AVIDIN SCREENING TEST FOR COCAINE AND BENZOYLECGONINE ON THE BECKMAN ARRAY® 360 SYSTEM.

Chan Oh, Janet Bui, Julie Kim, Josephine Michael, and Anthony Cheng (Beckman Instruments, Inc., Brea, CA 92621).

Clinical Chemistry (1994) Vol. 40, No. 6, pp. 633

A particle-based biotin-avidin screening test for cocaine and its major metabolite, benzoylecgonine, in urine samples has been developed. The assay utilizes a latex particle-avidin-biotinylated benzoylecgonine conjugate and a monoclonal antibody as the trigger reagent. When the reagents are mixed, agglutinated latex particles are produced and cause an increase in turbidity. The rate of the agglutination reaction is monitored on the Beckman ARRAY 360® system, an automated random-access nephelometer. Cocaine or benzoylecgonine inhibits the agglutination, and the extent of inhibition allows the quantitation of the drug in the urine sample.

Using a cut-off value of 0.15 μg/mL, the assay correctly predicted 84 (98.8%) of 85 urine samples confirmed to be positive by GC/MS (>0.15 μg/mL). Of 10 GC/MS confirmed negative samples, all were shown to be negative by this method. Within-run coefficients of variation (CV's) were <5% at 0.41 μg/mL and 1.6 μg/mL. Between-run CV's were 6.8% and 3.6% at 0.26 μg/mL and 0.73 μg/mL, respectively. The assay gave no false positive results when urine samples separately containing amphetamine (500 μg/mL), morphine (500 μg/mL), phencyclidine (500 μg/mL) and ecgonine (5 μg/mL) were analyzed. Sodium ion concentration up to 500 mM in urine sample did not interfere with the screening test.

The particle-based biotin-avidin rate nephelometric assay method developed for cocaine and benzoylecgonine on the Beckman Array® system is homogeneous, simple, fast, precise and easily adaptable to other hapten assays.

0234

## EVALUATION OF THE NEW RADIOIMMUNOASSAY FOR HUMAN C-PEPTIDE OF MEDGENIX

Iris Dotschkis, Gerald Steinbach, Michael Stein* and Volker Maier
Dept. of Internal Medicine, University of Ulm, and Medgenix Diagnostics*, Ratingen, Germany.

Human C-peptide (connecting peptide) originates as a by-product of the enzymatic splitting of pro-insulin to insulin. However, the half-life of C-peptide is ca. 30 min. compared to ca. 5 min of insulin. Measuring C-peptide it is possible to monitor insulin secretion in the presence of antiinsulin antibodies and under reduced insulin secretion resp. insulin therapy (exogenous insulin). It contributes to diagnosis of hypoglycaemia factitia and insulinoma (insulin suppression test).

The new method of MEDGENIX was compared with that of DIAGNOSTIC PRODUCTS CORPORATION. In the MEDGENIX procedure $^{125}$I Tyr-C-peptide competes with C-peptide in the sample for antibody sites immobilized on the wall of polystyrene tube for only 3 hours at room temperature. After aspiration and a washing step the tubes are aspirated again and measured. MEDGENIX uses the MRC 84/510 standard. Therefore the values determined are about three times higher. The minimal detectable dose both assays tested is approximately 0.22 ng/ml. Intra-assay and interassay CV are similar at 5.3 % resp. 7.2 %. The correlation coefficient is r = 0.91.

SUMMARY: The decisive technical advantage of the MEDGENIX test seems to be that it is 38 % more rapid and has one pipetting and centrifugation step less. However, both assays tested are outstanding radioimmunoassays helping in diagnosis and disease with fast and reliable results.

0235

## AN AUTOMATED CHEMILUMINESCENT IMMUNOMETRIC ASSAY FOR $\beta_2$-MICROGLOBULIN ON THE IMMULITE SYSTEM

P. Bodlaender, A. S. El Shami, J.-D Lei, H. Wilson (Diagnostic Products Corporation, Los Angeles, CA)

IMMULITE® Beta-2 Microglobulin is a solid-phase, two-site chemiluminescent immunometric assay developed for the IMMULITE – an automated, continuous random-access system. The assay is designed for measuring $\beta_2$-microglobulin ($\beta_2$M) in serum and urine as an aid in clinical diagnosis of active rheumatoid arthritis and kidney disease. The system pipets 10 μL of patient urine or (diluted) serum sample and 200 μL alkaline phosphatase (bovine calf intestine) conjugated to monoclonal anti-$\beta_2$M antibody into the proprietary Test Unit, which encloses a polystyrene bead coated with polyclonal goat anti-$\beta_2$M antibody. After a 30-minute incubation at 37°C, the bead undergoes four centrifugal washes, each time with 300 μL deionized water, waste is expelled into a sump which is an integral part of the Test Unit. The substrate, an adamantyl dioxetane phosphate ester solution, is added, and the enzyme activity is measured in a luminometer at 37°C. Results are calculated via a stored 4-parameter logistic Master Curve, which has a 4-week stability.

IMMULITE Beta-2 Microglobulin has a detection limit of 0.3 ng/mL (2SD above 0 dose), and a reportable range extending from 10 to 500 ng/mL. Intraassay CVs were 3.4 – 7.9% (means: 29 – 192 ng/mL) and interassay CVs were 7.0 – 10.1% (means: 28 – 198 ng/mL). The assay showed no detectable crossreactivity to other substances tested: in particular, human IgE was found to be nondetectable at concentrations as high as 5.0 g/L. Both linearity-under-dilution and spiking recovery experiments demonstrated good recoveries,

showed no high-dose

0236

## DEVELO ASSAY

Sherri L. Juveland (Abbott Laboratories, Abb

The TDx/TDxFLx Metho ment of methotrexate in ser immunoassay) technology. not recognize the 7-hydrox methotrexate cross-reactivi in managing patients on me

A brief description of the the calibration curve is 0 - methotrexate values can be calibrators are 0.00, 0.05, 0.80, 5.00, 50.00, and 500. depending on the protocol with a total of 24 minutes

Using the TDx/TDxFLx Precision: low control, 0.0 0.022 μmol/L (5.4% total control, 5.04 ± 0.28 μmol/ CV), and Z control, 495.16 obtained by following the (NCCLS) protocol. Recov sensitivity of the assay was not detected from anticoag The assay was linear throu within 10% from target in 0.09 μmol/L range. DAM 6 μmol/L. The 7-hydroxy- reactivity when tested up t assay versus Methotrexate intercept of -0.24 and a co Methotrexate II assay vers 1.01, a y intercept of -0.02

Thus, the TDx/TDxFLx method for monitoring pat

0237

## A NEW SCORING SYS

Ker-Kong Tung, Ru-L Inc., Garden Grove, C

Fadal and Nalebuff assay sensitivity (19 consistent under con system for the m-R reproduced their test components in the m-

In rapid EIA syster high concentration of of the calibration cur direct application of cause class-shift of t RAST test will read as

To eliminate this pr difference in shape of a monoclonal antibod combined improveme consistent performanc

Results of a correla RAST test on 361 dat + 0.028, r = 0.57

0238

## PRACTICABILITY AND PLUS AND BN 100

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As part of a clinical stuc lytical performance and assays on two systems which is a fluorogenic s a particle-enhanced IN comparison of OPUS

Correlation:

| Range | |
|---|---|
| < 80 ng/ml | 13 |
| < 600 | 2 |
| < 1000 | 27 |

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# Biotin-avidin immobilization of platelet glycoproteins (BAIPG): a new capture assay for the detection of anti-platelet antibodies

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**Abstract**

Several 'capture' assays are currently employed to identify specific platelet antibodies, but all require the use of murine monoclonal antibodies (MoAbs) against the antigen of interest. We have developed a new antigen capture assay for the detection of platelet reactive antibodies, based on platelet surface sialoglycoprotein labelling with biotin hydrazide, and a following immobilization of the biotinylated platelet proteins to microtiter wells that had been coated with streptavidin. The resulting solid phase can then be used in a simple ELISA to detect serum and platelet associated antibodies. We describe here two versions of this biotin-avidin immobilization of platelet glycoproteins (BAIPG) assay. In BAIPG assay type I, the test sera are directly incubated in microtiter wells previously coated with streptavidin plus biotinylated platelet proteins. The BAIPG type II procedure involves the incubation of sera with biotinylated platelets before platelet solubilization, and, after platelet lysis, the immobilization of the immune complexes to streptavidin-coated wells. In both cases, the bound antibodies are determined by alkaline phosphatase conjugated anti-human IgG. Using BAIPG type I, positive results were obtained in 7/33 patients with idiopathic thrombocytopenic purpura (ITP), 1/10 patients with secondary immune thrombocytopenia (SIT) and 4/17 with non-immune thrombocytopenia (NIT). The BAIPG type II test was positive in 13 out of 33 patients with ITP, in six out of ten patients with SIT, and in three out of the 17 patients with NIT. A comparison between BAIPG and monoclonal antibody immobilization of platelet antigens (MAIPA) assays showed a high degree of correlation between the two methods. These results suggest that the BAIPG assay is a valuable new tool for the detection of anti-platelet antibodies.

*Keywords:* Immune thrombocytopenia; Anti-platelet antibody; Biotin-avidin immobilization; Platelet glycoprotein

Abbreviations: IT, immune thrombocytopenia; ITP, idiopathic thrombocytopenic purpura; SIT, secondary immune thrombocytopenia; NIT, non-immune thrombocytopenia; BAIPG, biotin-avidin immobilization of platelet glycoproteins; MoAbs, murine monoclonal antibodies; MAIPA, monoclonal antibody immobilization of platelet antigens

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*SSDI* 0022-1759(94)00250-9

## 1. Introduction

Platelet destruction in immune thrombocytopenia (IT) is due to specific autoantibodies that bind to platelet surface antigens or to immune complexes which bind non-specifically to platelet Fc receptor.

The several assays to detect platelet antibodies can be divided into three general groups (Sinha and Kelton, 1990). Phase I assays are biological methods based on the demonstration of antibody-mediated platelet aggregation or lysis, but they lack both sensitivity and specificity for immune thrombocytopenia, and are no longer used. Phase II assays include immunological procedures, such as complement fixation, antiglobulin consumption, ELISA, RIA and immunofluorescence that measure platelet-associated (PAIgG) and platelet bindable (SPBIgG) immunoglobulins, and determine the amount of Ig bound or bindable to the surface of whole or lysed platelets. It is generally agreed that these assays have a high sensitivity but a low specificity for IT (Kelton et al., 1982; Mueller-Eckhardt et al., 1980) because they are inappropriate for distinguishing immune complexes from true antiplatelet antibodies. Phase III assays consists of immunoblotting, immunoprecipitation and 'capture' methods, that identify the specificity of platelet reactive antibodies. In the 'capture methods', human antibody bound to platelet glycoprotein is captured in the solid phase by murine monoclonal antibodies (MoAbs) specific for different epitopes of platelet surface glycoproteins. The major limitation of these techniques is the cost of the several monoclonal antibodies required and the presence of human natural antimouse immunoglobulins.

In this report, we describe a new capture procedure based on platelet surface glycoprotein labelling with a biotin derivative, and the subsequent immobilization of the biotinylated platelet proteins to microtiter wells by a specific interaction with streptavidin bound to the plastic. Therefore, only biotinylated platelet surface antigens are bound to the solid phase and can be used in an ELISA to detect serum and platelet associated antibodies. The biotin-avidin immobilization of platelet glycoproteins (BAIPG) assay described here requires small amounts of platelet proteins, does not require radiolabels or electrophoretic separation of platelet proteins, is simple to perform and is applicable to large-scale screening. With BAIPG, it is not possible to ascertain the specificity of anti-platelet antibodies, and the method is proposed as a screening capture assay before the application of phase III methods.

## 2. Materials and methods

### 2.1. Sera

Sera were obtained from 33 patients with idiopathic thrombocytopenic purpura (ITP), ten patients with secondary immune thrombocytopenia (SIT) (four lymphomas, five systemic lupus erythematosus, one systemic scleroderma), 17 patients with non-immune thrombocytopenia (NIT) and ten control subjects. Two HPA-1a antisera were used as positive controls.

### 2.2. Monoclonal antibodies

Murine monoclonal antibodies against GPIb$\alpha$ (LJ-P19, LJ-IB10), GPIIb/IIIa complex (LJ-P9, LJ-CP8) and GPIX (LJ-RA8) were used as purified heavy and light chain fractions; these monoclonal antibodies were a gift of Dr. Z.M. Ruggeri (Scripps Clinic, La Jolla, CA, USA) and have been previously characterised (Murata et al., 1991; Handa et al., 1986; Trapani Lombardo et al., 1985; Niiya et al., 1987). Murine monoclonal antibodies AN51 (specific for GPIb$\alpha$) and CLB-Thromb/1 (specific for GPIIIa (C17)) were purchased from Dakopatts (Glostrup, Denmark) and CLB (Amsterdam, Netherlands) respectively. The negative control consisted of murine monoclonal antibody OKB2 (CD24, Ortho Diagnostic System, Beerse, Belgium).

### 2.3. Platelet biotinylation

Platelets were labelled with biotinamidocaproyl hydrazide (BACH) as we previously de-

scribed (Fabris et al., 1992) with minor modifications. Briefly, sodium metaperiodate (Sigma Chemical Co., St. Louis, MO, USA) oxidation was blocked by adding PBS pH 7.3 (1:5, v/v), and the pellet was washed once with PBS/10 mM EDTA pH 7.3. Platelets ($10^9$/ml) were then incubated with 0.3 mM BACH (Sigma) for 60 min at 30°C. The labelled platelets were washed twice with PBS/10 mM EDTA pH 7.3 and resuspended in LPB (lysis platelet buffer) pH 7.4 (0.05 mM Tris-HCl, containing inhibitors: 10 mM EDTA, 5 mM NEM (*N*-ethyl-maleimide, Sigma), 1 mM PMSF (phenyl-methyl-sulfonyl fluoride, Sigma), and 200 μg/ml leupeptin (Sigma)). Biotin labelling was verified by SDS-PAGE separation of biotinylated platelet glycoproteins (50 μg of total platelet proteins), and immunoblotting, as previously described (Fabris et al., 1992).

### 2.4. Preparation of microtiter plates

50 μl of streptavidin (14 U/mg, Sigma) in 0.05 mM carbonate buffer (pH 9.5) at a concentration of 10 μg/ml were added to each well of a 96-well flat-bottom microtiter plate (Costar, Cambridge, USA), and incubated overnight at 4°C. The wells were washed three times with washing buffer (PBS with 0.05% Tween 20, 0.5% Nonidet P40 (NP40), and 0.5 mM $CaCl_2$), and blocked with the same buffer for 30 min at 37°C. The plate was then washed three times with the buffer and streptavidin binding was determined by peroxidase-diaminodipropylamine (DAPA)-biotin (Sigma). Biotinylated platelets were solubilized in LPB containing 1% NP40 and stirred for 30 min at 25°C. The insoluble material was removed by ultracentrifugation at 100000 × *g* for 30 min at 4°C, and the supernatant stored at −80°C. Biotinylated platelet proteins were diluted in washing buffer immediately before use, and 50 μl (2.5 μg total platelet proteins/well) were added to even numbered columns (test wells); washing buffer alone was added to the odd numbered columns (blank wells). After incubation for 30 min at 25°C, the plate was washed three times, and stored at 4°C for 2 months (the longest time tested without loss of binding activity).

### 2.5. Biotin-avidin immobilization of platelet glycoproteins (BAIPG) type I

The MoAbs were used at the following concentrations: 1.3 μg/ml LJ-P19, 2.5 μg/ml LJ-P9, 2.5 μg/ml LJ-CP8, 2.6 μg/ml LJ-IB10, 7.0 μg/ml LJ-RA8, 2.0 μg/ml AN51, 4.0 μg/ml CLB-Thromb/1, and 1.0 μg/ml OKB2. Test and control sera were diluted 1/10 and 1/50 in washing buffer. 50 μl of MoAbs, control and test sera were added to the wells. After incubation for 60 min at 25°C, the plate was washed six times with washing buffer. 50 μl of the second antibody (alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) or (Sigma) sheep anti-mouse IgG (whole molecule) diluted 1/10000 and 1/1000 respectively, in PBS pH 7.4 containing 1% bovine serum albumin (BSA)) were added and the plate was incubated for 60 min at 25°C. After six additional washes, 100 μl of *p*-nitrophenylphosphate (1 mg/ml in 0.05 mol/l carbonate buffer pH 9.8 containing 0.5 mmol/l $MgCl_2$) were added to each well. After 60–180 min at 25°C, the reaction was then stopped by 50 μl of 3 N NaOH and optical density (OD) was read at 405 nm (photometer Titertek-Multiskan, Flow Labs., Irvine Scotland, UK). For each sample the OD was expressed as the difference between the OD of the test well and the OD of the control well. All sera were run in duplicate. Results were expressed as ΔE values, i.e., the difference in extinction (OD) values between the mean of test samples and blanks.

### 2.6. Biotin-avidin immobilization of platelet glycoproteins (BAIPG) type II

Whole biotinylated platelets were resuspended to 100000/μl in PBS pH 7.4 containing 2% BSA. $20 \times 10^6$ platelets were then centrifuged at 10000 × *g* for 1 min and the platelet pellet was resuspended in 50 μl of test serum. After incubation at 37°C for 30 min, 100 μl of PBS/BSA were added, and the samples were centrifuged for 1 min at 10000 × *g*. Platelets were then washed three times with PBS/BSA and solubilized with 50 μl of TBS pH 7.4 (20 mmol/l Tris, 0.15 mol/l

NaCl) containing 0.5% NP40. After 30 min incubation at 4°C, the platelets were centrifuged at 10000 × g for 30 min at 4°C and the supernatant was diluted 1/16 in washing buffer. 50 μl of the diluted supernatant were added to streptavidin-coated wells and the microtiter plate was incubated for 30 min at 25°C; after three washes, the ELISA was performed as described above for BAIPG type I.

### 2.7. Monoclonal antibody immobilization of platelet antigens (MAIPA)

The MAIPA assay was performed as previously described with some modifications (Kiefel et al., 1987). Platelets were washed three times in Tyrode buffer pH 6.5 supplemented with $PGE_1$ (9 ng/ml, Sigma) and resuspended in PBS/2% BSA at 100000/μl. 200 μl of platelet suspension were centrifuged at 10000 × g for 1 min, the pellet was then resuspended in 50 μl of test serum, and 50 μl of anti-GPIbα and anti-GPIIb-IIIa specific MoAb (20 μg/ml LJ-IB10 and 10 μg/ml LJ-CP8) were added. The platelets were then solubilized in TBS/0.5% Triton X-100 for 30 min at 4°C. After centrifugation at 10000 × g for 30 min at 4°C, the supernatant was diluted 1/4 with TBS washing buffer (TBS supplemented with 0.5% Triton X-100, 0.05% Tween 20 and 0.5 mmol/l $CaCl_2$) and 100 μl were added to each well of a microtiter plate that had been coated with 100 μl of goat anti-mouse IgG (7.5 μg/ml). After incubation at 4°C for 90 min the plate was washed three times, and 100 μl of horseradish peroxidase-conjugated goat anti-human IgG (Caltag, South San Francisco, Ca, USA) at a 1/3000 dilution in PBS/2% BSA were added to each well and the plate was incubated for 120 min at 4°C. After five additional washes, 100 μl of OPD (0.5 mg/ml *o*-phenylenediamine dihydrochloride in 0.05 M phosphate-citrate buffer pH 5.0) were added. The color reaction was then stopped with 50 μl of 2 N $H_2SO_4$ and OD was determined at 492 nm in a Titertek photometer. All sera were run in duplicate. Results were expressed as ΔE values, i.e., the difference in extinction (OD) values between the mean of test samples and blanks.

## 3. Results

### 3.1. Platelet biotinylation

Three main platelet surface glycoproteins were observed on immunoblots of biotinamidocaproyl hydrazide-labelled platelets. The relative molecular weights were 165 kDa,135 kDa and 88 kDa which corresponded to the molecular weight of GPIbα, GPIIb and GPIIIa (Fig. 1).

### 3.2. Assay conditions

To determine the optimal conditions for coating streptavidin to the plastic wells, streptavidin (1 μg/ml) was diluted in PBS pH 5.0, PBS pH 7.4 and 0.05 mM carbonate buffer pH 9.5, respectively. Streptavidin binding to the plastic surface was determined by ELISA using peroxidase-DAPA-biotin. The results of this preliminary experiment showed that the carbonate buffer per-

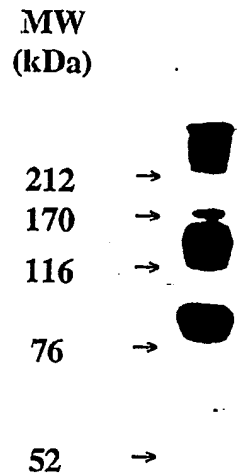


Fig. 1. Fluorography pattern of biotin hydrazide-labeled platelet proteins (50 μg) separated under non-reducing conditions by SDS-PAGE (7.5%) and transblotted onto nitrocellulose sheets. The immunoblot was neutralized, washed and incubated with avidin-peroxidase and a light-emitting substrate (luminol-4-iodophenol) followed by contact exposure with X ray film.

mitted the greatest binding of streptavidin to the microtiter plates.

The optimal amounts of streptavidin and platelet lysate was defined using increasing amounts of streptavidin (0.1–2 μg/well) and platelet lysate (1–20 μg total platelet proteins/well). Coating of the platelet glycoproteins was assayed by anti-GPIb and GPIIb/IIIa (LJ-P19, LJ-P9) MoAbs and control murine antiserum (OKB2). By increasing the amount of streptavidin and platelet lysate, the optical density as well as the background increased. We then chose 0.5 μg streptavidin/well and 2.5 μg total platelet proteins/well as standard conditions.

We used PBS/0.5% NP40/0.05% Tween 20/0.5 mM $CaCl_2$ buffer to block both the unbound sites of the microtiter plates and wash the wells, since its use afforded a better differentiation between positive and negative samples. The use of 1–2 mg/ml bovine serum albumin in the blocking buffer did not improve the signal-to-noise ratio and even increased the non-specific binding.

### 3.3. Platelet glycoprotein binding

The antigenicity and the binding of biotinylated glycoproteins to the immobilized streptavidin were tested by murine MoAbs against the main platelet-surface glycoproteins. We tested LJ-P19, LJ-IB10 and AN51 (anti-GPIbα), LJ-P9 and LJ-CP8 (anti-GPIIb/IIIa complex), CLB-Thromb/1 (anti-GPIIIa), LJ-RA8 (anti-GPIX) and OKB2 (CD24, as negative control). Each MoAb was run in both blank wells (streptavidin only), test wells (streptavidin plus biotinylated platelet proteins) as shown in Table 1. The ΔE between test and blank wells was 1.368 (LJ-P19), 0.487 (AN51), and 0.270 (LJ-IB10) using MoAbs against GPIb. Anti-GPIIb/IIIa MoAbs showed ΔE values of 0.610 (LJ-CP8) and 0.570 (LJ-P9), respectively. Using MoAbs specific for GPIIIa (CLB-Thromb/1) and GPIX (LJ-RA8) the ΔE values were 0.310 and 0.400, respectively.

The ELISA gave a low signal when biotinylated or non-biotinylated platelet proteins were coated directly to the wells lacking streptavidin or when the wells coated with streptavidin were incubated with non-biotinylated platelet proteins (data not shown). These experiments showed that the formation of the streptavidin-biotin complex was essential for the immobilization of platelet antigens to the solid phase.

### 3.4. BAIPG type I

Positive control sera included two anti-HPA-1a antisera with different amounts of anti-platelet antibody. A titration experiment is shown in Fig. 2.

Anti-platelet antibodies were demonstrable in seven out of 33 ITP patients (21%), one out of ten SIT patients (10%) and four out of 17 NIT patients (24%). 21 sera were negative while inconsistent findings were observed in 27 (45%) of the 60 patients due to a high binding of serum IgG to the blank wells, as shown in Table 2. This non-specific binding was observed even when

Table 1
Binding of anti-platelet monoclonals to biotinylated sialoglycoproteins immobilized to streptavidin-coated microtiter wells

| | MoAbs (μg/ml) | Blank well (streptavidin) (OD 405 nm) | Test well (streptavidin + biotinylated platelet proteins) (OD 405 nm) |
|---|---|---|---|
| Blank | | 0.156 | 0.220 |
| OKB2 (negative control) | 1.0 | 0.237 | 0.318 |
| LJ-P9 (anti GPIIb-IIIa) | 2.5 | 0.187 | 0.757 |
| LJ-CP8 (anti-GPIIb-IIIa) | 2.5 | 0.115 | 0.735 |
| LJ-P19 (anti-GPIbα) | 1.3 | 0.156 | 1.524 |
| LJ-IB10 (anti-GPIbα) | 2.6 | 0.139 | 0.410 |
| LJ-RA8 (anti-GPIX) | 7.0 | 0.200 | 0.610 |
| AN51 (anti-GPIbα) | 2.0 | 0.106 | 0.593 |
| CLB-THR.1 (anti-GPIIIa) | 4.0 | 0.115 | 0.425 |

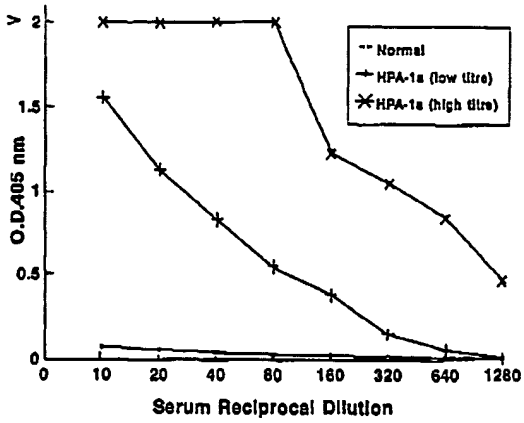


Fig. 2. Reactivity of normal serum and two anti HPA-1a sera in the BAIPG assay type I. Sera were applied to microtiter wells previously coated with streptavidin plus biotinylated platelet surface sialoglycoproteins. Binding was monitored by ELISA using alkaline-phosphatase conjugated goat anti-human IgG (Fc specific) and the appropriate substrate. OD was read at 405 nm.

Table 2
Optical density values at 405 nm obtained by BAIPG assay type I using control sera and the sera of nine patients with ITP 1/10 diluted

| | Blank well (streptavidin) | Test well (strept.+ biotinylated platelet proteins) |
|---|---|---|
| Blank | 0.051 ± 0.032 (M ± SD) [a] | 0.068 ± 0.058 |
| Normal serum | 0.133 ± 0.019 | 0.206 ± 0.058 |
| HPA-1a | 0.189 ± 0.047 | 1.384 ± 0.253 |
| ITP 4 | 0.857 [b] | 0.950 |
| ITP 6 | 1.187 [b] | 1.275 |
| ITP 8 | 1.014 [b] | 0.916 |
| ITP 10 | 0.617 [b] | 0.522 |
| ITP 14 | 0.171 | 0.425 [c] |
| ITP 2 | 0.360 | 0.670 [c] |
| ITP 23 | 0.511 | 1.043 [c] |
| ITP 24 | 0.171 | 0.637 [c] |
| ITP 25 | 0.137 | 1.278 [c] |

ITP 4-6-8-10 sera showed a high OD signal in control wells. ITP 14-21-23-24-25 sera showed a high OD value in test wells only and were considered as positive.
[a] Mean ± standard deviation of five different experiments.
[b] High background.
[c] Positive sample.

streptavidin was omitted and thus should be due to the direct binding of serum IgG to the plastic surface. The use of albumin in the blocking or washing buffer and the use of different plastics did not reduce this binding.

### 3.5. BAIPG type II

In BAIPG type II, biotinylated platelets were sensitised with serum before platelet solubilization and the immune complex was then immobi-

Table 3
Optical density values obtained by BAIPG assay type II using control sera, sera of seven patients with ITP (1/10 diluted) and monoclonals against platelet glycoproteins Ib and IIb-IIIa

| | Optical density 405 nm | | Optical density 405 nm |
|---|---|---|---|
| Blank | 0.111 ± 0.011 (M ± SD) [a] | Blank | 0.102 ± 0.015 |
| Normal serum | 0.139 ± 0.028 | OKB2 (neg. control) 1 μg/ml | 0.105 ± 0.020 |
| HPA-1a low titre | 0.760 ± 0.059 | LJ-P9 (anti-GPIIb-IIIa) 10 μg/ml | 0.562 ± 0.039 |
| HPA-1a high titre | 2.192 ± 0.293 | LJ-CP8 (anti-GPIIb-IIIa) 10 μg/ml | 0.474 ± 0.033 |
| ITP 4 | 0.455 | LJ-P19 (anti-GPIbα) 20 μg/ml | 0.404 ± 0.028 |
| ITP 6 | 0.697 | | |
| ITP 8 | 0.508 | | |
| ITP 10 | 0.331 | | |
| ITP 14 | 0.455 | | |
| ITP 21 | 0.195 | | |
| ITP 23 | 0.391 | | |
| ITP 24 | 0.453 | | |
| ITP 25 | 0.261 | | |

The alkaline-phosphatase conjugated second antibodies were respectively goat anti-human and sheep anti-mouse IgG.
[a] Mean ± standard deviation of five different experiments.

lized to the streptavidin-coated wells. The constitution of the trimolecular complex formed by platelet reactive antibody, biotinylated glycoproteins and streptavidin was verified using two anti-HPA-1a antisera with different amounts of anti-platelet antibody, normal and patient sera or murine MoAbs against the platelet GP Ib and IIb-IIIa (Table 3). Anti-platelet antibodies were demonstrated in 13 out of 33 ITP patients (39%), in six out of ten SIT patients (60%) and in three out of 17 NIT patients (17%). The prevalence of reactive platelet antibodies in the 43 patients with immune thrombocytopenia was 44%.

### 3.6. MAIPA

The MAIPA assay was performed using MoAbs specific for GPIb (LJ-IB10) and GPIIb/IIIa complex (LJ-CP8). Reactive platelet antibodies were detected in 16 (37%) out of 43 patients with immune thrombocytopenia. Among ITP patients, four had anti-GPIb antibodies, five had anti-GPIIb/IIIa antibodies, and three had both types of antibody. Among the SIT patients, three had anti-GPIb antibodies and one had anti-GPIb and GPIIb/IIIa antibodies. Only one out of 17 patients with NIT had anti-GPIIb/IIIa antibodies.

The association between the positive and negative results obtained by BAIPG type I and II and MAIPA assays was tested using the $\chi^2$ method (Tables 4 and 5).

The association between BAIPG type I and II reached statistical significance the sera with high backgrounds when tested by BAIPG type I were included as positive (BAIPG type I/type II: $\chi^2 = 10.25$ $p < 0.002$). The relationship between MAIPA and BAIPG type II was also statistically significant (MAIPA/BAIPG type II: $\chi^2 = 8.03$ $p < 0.005$).

Table 4
Contingency table of the results obtained using BAIPG type I and type II assays for the detection of serum anti-platelet antibodies

| BAIPG type II | BAIPG type I | | Total |
|---|---|---|---|
| | Positive [a] | Negative | |
| Positive | 20 | 2 | 22 |
| Negative | 19 | 19 | 38 |
| Total | 39 | 21 | 60 |

[a] Including high background samples; $\chi^2 = 10.25$; $p < 0.002$.

Table 5
Contingency table of the results obtained using BAIPG type II and MAIPA assays for the detection of serum anti-platelet antibodies

| MAIPA | BAIPG type II | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 11 | 6 | 17 |
| Negative | 11 | 32 | 43 |
| Total | 22 | 38 | 60 |

$\chi^2 = 8.03$; $p < 0.005$.

## 4. Discussion

None of the assays devised to detect anti-platelet antibodies have been fully satisfactory, nevertheless, the recently developed procedures have shown that GPIIIa (Van Leeuwen et al., 1982), GPIIb (Tomiyama et al., 1987), GPIb (Woods et al., 1984) and GP V (Stricker and Shuman, 1986) carry the main antigens involved in platelet immune destruction.

The techniques based on solid-phase immobilization of platelet glycoproteins by MoAbs represent the newer generation of tests to detect specific anti-platelet antibodies. The sensitivity of these assays in the detection of plasma autoantibodies is about 60% (McMillan, 1990). Several, so called 'capture' assays, are currently in use: (1) the monoclonal immobilization of platelet antigens assay (MAIPA) (Kiefel et al., 1987); (2) the immunobead assay (McMillan et al., 1987); (3) the modified antigen capture ELISA (MACE) (Ishida et al., 1991) and (4) the bead-immobilized platelet antigen (BIPA)-antibody assay (Loliger et al., 1993), but all are variations on a theme. Although these tests are simple to perform, they require MoAbs against the different glycoproteins and thus only detect antibody reactivity against those glycoproteins for which MoAbs are available; this constitutes a limitation.

We describe a new capture assay based on the specific interaction of biotinylated platelet glycoproteins with streptavidin-coated microtiter plates. Streptavidin's interaction with biotin is characterised by the highest association constant yet reported ($K_a = 10^{15}\ M^{-1}$) (Green, 1975). This interaction is so strong that the protein-linked biotin is available for binding to avidin (Wilchek and Bayer, 1990) and the complex is not disturbed by multiple washings. The streptavidin-biotin complex can then be used as a bridge system to immobilise the platelet proteins in a solid phase that can be used in an ELISA procedure to measure platelet-specific antibodies.

Platelet membrane sialoglycoproteins can be easily biotinylated using $NaIO_4$ to convert sialic acid residues into aldehyde groups, which are then allowed to react with biotin hydrazide. Immunoblots of biotin hydrazide-labelled platelet glycoproteins revealed satisfactory resolution of GPIb, GPIIb and GPIIIa.

Streptavidin immobilization to the solid phase and the following binding with biotinylated platelet antigens are the prerequisites for platelet antibody detection by the BAIPG assay. In fact, a low signal was generated in ELISA when biotinylated or non-biotinylated platelet proteins were coated directly to the plastic wells and similar results were obtained when non-biotinylated proteins were added to the wells coated with streptavidin. As previously described (Fabris et al., 1992), biotinylation does not modify the antigenic properties of the surface glycoproteins that via biotin can bind to streptavidin-coated wells and react with the antibodies. Low amounts of glycoproteins are used in our BAIPG assay; indeed by adding 2.5 $\mu$g of platelet proteins to the microtiter well coated with 0.5 $\mu$g of streptavidin, about 50 ng of biotinylated platelet glycoproteins are immobilized. The platelet glycoproteins Ib$\alpha$, IIb, IIIa, and IX that bear the main platelet specific antigens were immobilized to the solid phase as shown by the binding of specific anti-platelet MoAbs.

In type I BAIPG, control and patient sera were tested directly against platelet glycoproteins that were previously immobilized to streptavidin coated wells. To avoid false positives due to non-specific binding to the plastic surface, we tested each serum, in a blank well coated only with streptavidin and in a test well coated with streptavidin plus biotinylated platelet proteins. Using BAIPG type I, positive results were obtained in 19% of patients suffering from immune thrombocytopenia while 45% of the sera yielded high absorbance values in both blank and test wells. In the immunoassay procedure, the non-specific binding of antibodies to reactive plastic sites is generally reduced by addition of bovine serum albumin in blocking and sample dilution buffers. Our data showed that the use of serum bovine albumin did not eliminate the background and suggested that albumin was even harmful. Similar observations were recently reported by Landgraf et al. (1991). The addition of detergents to the buffer led to a reduction of non-specific binding even if its use did not completely eliminate the background. We also considered whether the high non-specific binding might be due to the presence of IgG aggregates in sera stored at −20°C for prolonged periods of time (more than 1 year) as reported by Karpatkin et al. (1992). However, neither serum storage at 4°C nor its filtration through a 0.22 $\mu$m filter influenced the non-specific binding.

To avoid the binding of serum IgG to the plastic surface, we performed the BAIPG type II assay. In this assay the sera were incubated with biotinylated platelets before platelet lysis and the immunocomplex was then immobilized to streptavidin coated wells. This approach permitted a sensitive differentiation between negative and positive samples.

Using the BAIPG type II assay, positive results were obtained in 44% of patients suffering from immune thrombocytopenia. There are two different explanations for the greater positivity obtained by BAIPG type II. First, the sensitisation of whole platelets with the antibodies prevents the loss of the epitopes which might be destroyed during platelet lysis (McMillan et al., 1987). Second, the washes after platelet sensitisation remove the 'non-specific' IgG that interfere with the BAIPG type I. However 16 out of 27 sera (59%) which showed high background activity when tested by BAIPG type I, were positive using

BAIPG type II and the association between these two assays reached statistical significance when the sera with high background were included as positive.

Like the 'capture' assays, BAIPG type II is based on the detection of trimolecular complexes formed by platelet reactive antibody, biotinylated sialoglycoproteins and streptavidin. The percentage of positivity obtained testing sera of patients with immune thrombocytopenia by BAIPG type II was comparable to that obtained by others capture assays (McMillan, 1990) and was significantly related to the results obtained with the MAIPA reference method. The BAIPG assay is rapid, specific, reproducible and does not require the use of expensive monoclonal reagents. However, since the positive result obtained with this procedure requires further characterisation of the antibody specificity by MAIPA, immunoblot or immunoprecipitation, the method is proposed as a screening capture assay before the use of phase III methods.

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| L4 | vancomycin same l2 | 1 | L4 |
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